

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 March 2001 (01.03.2001)

PCT

(10) International Publication Number  
**WO 01/14580 A1**

(51) International Patent Classification<sup>7</sup>: C12Q 1/00, 1/04, 1/68, G01N 33/53, 33/567, 33/542, 33/537, 33/543

(21) International Application Number: PCT/US99/19418

(22) International Filing Date: 25 August 1999 (25.08.1999)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant (for all designated States except US): ACCU-  
PLEX, L.L.C. [US/US]; 9967 Florence Heights Boule-  
vard, Omaha, NE 68112 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): MCDONALD,  
Thomas, L. [US/US]; 9967 Florence Heights Boulevard,  
Omaha, NE 68112 (US). WEBER, Annika [US/US]; 802  
N. 72nd Street, Omaha, NE 68114 (US).

(74) Agent: NEBEL, Heidi, S.; Zarley, McKee, Thomte,  
Voorhees & Sease, P.L.C., Suite 3200, 801 Grand Avenue,  
Des Moines, IA 50309-2721 (US).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,  
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,  
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,  
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO,  
RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG,  
US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM,  
AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT,  
BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,  
NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA,  
GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.



**WO 01/14580 A1**

(54) Title: DIAGNOSTIC ASSAYS OF SECRETED BIOLOGICAL FLUIDS FOR DETECTION OF INFECTION AND IN-  
FLAMMATORY CONDITIONS

(57) Abstract: A rapid and convenient method for detecting inflammatory conditions in breast tissue, such as those associated with mastitis, is described. The method comprises measuring the presence and quantity of Serum Amyloid A (SAA) in milk samples obtained from the breast tissue. The amount of SAA present in the milk is positively correlated with the level of inflammation of the breast tissue, and can localize the inflammation to a particular region of the breast organ, such as a specific quadrant of a cow's udder. Test kits and their use in the method are also described.

**DIAGNOSTIC ASSAYS OF SECRETED BIOLOGICAL FLUIDS FOR  
DETECTION OF INFECTION AND INFLAMMATORY CONDITIONS**

**FIELD OF THE INVENTION**

The present invention relates generally to the field of diagnostics. In particular, the invention provides a method for diagnosing infection and other  
5 inflammatory conditions by measuring the presence of Serum Amyloid A in a secreted biological fluid. The invention further provides a test kit for use in the method.

**10 BACKGROUND OF THE INVENTION**

Several scientific or patent publications are referenced in this patent application to describe the state of the art to which the invention pertains. Each of these publications is incorporated by reference  
15 herein, in its entirety.

Mammals respond to tissue injury, trauma or infection by executing a complex series of biological reactions in an effort to prevent further tissue damage, to initiate repair of damaged tissue, and to isolate and  
20 destroy infective organisms. This process is referred to as the inflammatory response, the early and intermediate stages of which are referred to as the acute phase response.

The acute phase response involves a wide  
25 variety of mediators, including cytokines, interleukins and tumor necrosis factor. It also involves a radical alteration in the biosynthetic profile of the liver. Under normal circumstances, the liver synthesizes a range of plasma proteins at steady state concentrations. Some  
30 of these proteins, the "acute phase" proteins are induced

in the inflammatory response to a level many times greater than levels found under normal conditions. Acute phase proteins are reviewed by Steel & Whitehead (Immunology Today 15: 81-87, 1994).

5               One of the massively induced acute phase proteins is Serum Amyloid A (SAA). SAA actually comprises a family of polymorphic proteins encoded by many genes in a number of mammalian species. SAAs are small apolipoproteins that accumulate and associate  
10 rapidly with high-density lipoprotein 3 (HDL3) during the acute phase of the inflammatory response. Most SAAs are induced in response to inflammation; however, certain SAAs (e.g., human SAA4) appear to be constitutively expressed or minimally induced in the inflammatory  
15 response.

SAAs are regulated transcriptionally and post transcriptionally, though transcriptional regulation appears to predominate. SAA mRNA levels have been observed to increase up to 1,000 fold in the hours  
20 following an inflammatory stimulus. Likewise, plasma concentrations of SAA protein have been shown to increase as much as 1,000 fold, to levels approaching 1 mg/ml, for short periods following an inflammatory stimulus.

The massive increase in SAA plasma levels in  
25 response to both infective and non-infective inflammatory stimuli has led to its use as a diagnostic marker of inflammation. Among the most effective assays are immunoassays utilizing antibodies raised in a species that does not produce detectable amounts of SAA. For  
30 instance, McDonald et al. (J. Immunol. Meth. 144: 149-155, 1991) describe an antibody sandwich assay using two purified rat monoclonal antibodies raised against human SAA. Immunoassays utilizing these antibodies were demonstrated to be reliable and sensitive, and do not  
35 require denaturation of the specimen prior to assay (McDonald et al., 1991, *supra*). Similarly, Satoh et al.

(Am J. Vet. Res. 56: 1286-1291, 1995) describe an ELISA assay for measuring SAA levels in horse serum using rabbit anti-horse SAA antibodies. Though effective, these and similar immunoassays are invasive in that they  
5 require a blood sample. Moreover, they may not be appropriate or effective for early detection of localized inflammation, which is common in connection with a variety of infectious and non-infectious tissue trauma.

One excellent example of a non-systemic  
10 inflammatory-related disease of great economic importance to the dairy industry is mastitis. Mastitis is generally regarded as an inflammation of the mammary gland. The disease can affect any mammal, but is most economically significant in dairy heifers and cows. Mastitis usually  
15 results from colonization of the mammary gland by pathogenic bacteria. However, physical injuries or local mechanical or chemical stresses in the udder can also trigger a local inflammation cascade without the involvement of any primary bacterial infection (sometimes  
20 referred to as sterile mastitis).

Mastitis can be expressed at clinical or subclinical levels, and may be localized to only a portion of the udder. Subclinical or localized mastitis is economically damaging because it often remains  
25 undetected and untreated, yet results in decreased milk production. Accordingly, it is important in the early diagnosis of mastitis to be able to detect infection before clinical symptoms arise, and to be able to localize the infection to specific regions of the udder.

30 Current clinical laboratory methods used for the diagnosis of mastitis include estimation of somatic cell counts (SCC), various electrolyte levels, and soluble proteins, such as lactate dehydrogenase (LDH) and N-acetyl- $\beta$ -D glucosamidase (NAG), in milk samples, all of  
35 which reflect a breakdown in the blood-milk barrier due to the inflammatory response caused by the infection.

Certain of these parameters, e.g., SCC and electrolyte estimates, are unable to differentiate infected from uninfected regions of the udder (Zank & Slatterer, J. Vet. Med. 45: 41-51, 1998), while others, e.g., LDH and  
5 NAG detection, may not be sufficiently sensitive for very early diagnosis. Accordingly, new indicators that are highly predictive of the onset of mastitis infection at an early stage are needed.

## 10 SUMMARY OF THE INVENTION

According to one aspect of the invention, a rapid and convenient method for detecting an inflammatory response in a breast of a lactating mammal is provided. The method comprises measuring the presence or amount of  
15 a Serum Amyloid A (SAA) protein or mRNA encoding the protein, in a sample of milk obtained from the breast, the amount of the SAA protein or mRNA present in the sample being positively correlated with the inflammatory response. In a preferred embodiment, the inflammatory  
20 response reflects mastitis in the animal, caused either by infection with a pathogenic organism or by a non-infective stress or trauma to the breast tissue.

The SAA detected and quantified in the milk preferably comprises one or more inflammation-responsive  
25 isoforms of SAA. Specifically, the SAA comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 1 - 15.

In one embodiment, the method comprises measuring the amount of SAA protein in the milk sample.  
30 Preferably, the amount of SAA protein is measured using an immunological assay with antibodies immunologically specific for one or more isoforms of SAA. Most preferably, the immunological assay is an ELISA assay.

Another embodiment comprises measuring the

amount of SAA mRNA in the milk sample. Preferably, the amount of SAA mRNA is measured using a hybridization assay with nucleic acid molecules complementary to the SAA mRNA.

5                   The methods of the invention may be adapted for a variety of purposes. In one embodiment, a method is provided for evaluating a therapeutic agent or treatment for its ability to decrease or increase the inflammatory response in breast tissue. In this embodiment, the  
10   therapeutic agent or treatment is applied to a test subject. A milk sample is taken from the test subject and from a control subject that did not receive the agent or treatment. SAA levels in the test sample and the control sample are compared. An increase in SAA amount  
15   in the test sample as compared with the control sample indicates that the therapeutic agent has a detrimental side effect of increasing inflammation in the breast tissue. A decrease in SAA in the test sample as compared with the control sample indicates that the therapeutic  
20   agent or treatment decreases inflammation in the breast tissue.

                  In another embodiment, the methods of the invention are employed to monitor milk quality. Samples of milk batches are assayed for the presence and amount  
25   of SAA, and compared to a sample accepted as a known standard for a pre-determined milk quality. The comparative amount of SAA in the test sample versus the standard is used to assign a milk quality rating to the batch of milk.

30                   In another embodiment, the methods of the invention are used to determine the presence and amount of colostrum in a milk sample. This embodiment makes use of the inventors' discovery that SAA levels are elevated in colostrum, but not in normal milk. Test samples of

milk batches suspected of containing colostrum are assayed for SAA (preferably a colostrum-specific SAA) and compared to a sample known to be colostrum-free. The comparative amount of SAA in the test sample versus the control sample is determinative of whether the test sample is tainted with colostrum.

According to another aspect of the invention diagnostic kit for screening milk samples to detect inflammation in breasts of lactating mammals are provided. In one embodiment, a test kit comprises a container containing one or more antibodies immunologically specific for one or more SAA isoforms, and instructions for performing immunological assays of milk samples for SAA, using the antibodies. In another embodiment, the test kit comprises a container containing one or more nucleic acid molecules that specifically hybridizes to mRNA encoding or more SAA isoforms, and instructions for performing hybridization assays of milk samples for SAA, using the nucleic acids. These kits may further comprise at least one additional reagent for performing the immunologic or hybridization assays.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed descriptions and examples that follow.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

Various terms relating to the methods and kits of the present invention are used hereinabove and also throughout the specification and claims.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but

which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides or other  
5 single-stranded nucleic acid molecules, the term  
"specifically hybridizing" refers to the association  
between two single-stranded nucleic acid molecules of  
sufficiently complementary sequence to permit such  
hybridization under pre-determined conditions generally  
10 used in the art (sometimes termed "substantially  
complementary"). In particular, the term refers to  
hybridization of an oligonucleotide with a substantially  
complementary sequence contained within a single-stranded  
DNA or RNA molecule, to the substantial exclusion of  
15 hybridization of the oligonucleotide with single-stranded  
nucleic acids of non-complementary sequence.

## II. Description

Serum amyloid A (SAA) is an acute phase protein  
which is produced in the liver and occurs at elevated  
20 levels in the serum of mammals in response to  
inflammation related to tissue injury or infection. The  
present invention arises from the surprising discovery  
that elevated levels of SAA also occur in the milk of  
lactating females, in response to inflammatory conditions  
25 of the breast. In particular, cows with mastitis display  
elevated SAA levels that correlate with the severity of  
the infection, and that are confined to the area of the  
udder affected with the mastitis associated inflammation.  
This is believed to be the first observation of increased  
30 production of SAA in a secreted biological fluid, in  
response to inflammation of the tissue in which that  
fluid is produced.

Without being limited by any particular  
explanation, the SAA found in milk from inflamed breast  
35 tissue may result in part from a breakdown in the blood-



milk barrier, allowing cellular material and plasma enzymes to leak into the milk. However, experimental results obtained by the inventors show that serum SAA levels in cows is independent of colostrum and whey SAA levels. In samples of colostrum, whey and serum taken from test cows, serum SAA was found to be in the range of 10  $\mu\text{g/ml}$ , while in colostrum, SAA was elevated to levels in the range of 470  $\mu\text{g/ml}$  (average of 5 cows). These results indicate that the source of the SAA in milk arises from an independent source in the mammary tissue, e.g., ductal epithelial cells.

The measurement of SAA levels in milk for early detection of inflammation related to mastitis is exemplified herein. However, it will be apparent to persons of skill in the art that the principles applied to SAA detection in milk to diagnose mastitis-related inflammation likewise can be applied to detection of inflammatory conditions caused by other agents, such as mechanical injury, granulomatous disease, fibrocystic disease and cancer.

Furthermore, if SAA is detected in milk as an indicator of an inflammation in the tissue from which it was secreted, it is likewise expected to be found in other biological fluids secreted from inflamed tissue. For instance, elevated SAA levels are expected to occur in urine from an infected kidney or bladder. Elevated SAA levels may also occur in saliva, sputum, sweat or tears, arising from infected oral, lung, skin or lachrymose tissues, respectively. Application of the methods described herein for early detection of inflammatory conditions of those tissues are considered within the scope of the present invention.

As one example, SAA content in sputum may be used to identify a variety of lung inflammatory conditions in a patient (human or animal), including fungal and bacterial infections of the lung, as in

hypersensitive pneumonitis (e.g., Farmer's lung), viral and bacterial pneumonia, bacterial colonization and growth in Cystic Fibrosis flare (primarily caused by *Pseudomonas*), and cancer. Such application is considered particularly useful in equine veterinary applications, where unspecified or sub-clinical lung inflammations can undermine the general health of valuable horses, such as racehorses (see, e.g., Equine Veterinary Journal 21:106-109, 1989; Journal Veterinary Medical Science 55: 1011-1116, 1993).

As another example, SAA content in urine may be used to identify a variety of kidney or bladder inflammatory conditions in a patient (human or animal), including interstitial cystitis (bladder or kidney involved) and kidney transplant rejection.

Referring now to the exemplary embodiment of the invention, milk is analyzed for the presence and amount of SAA as a diagnostic test for inflammation, usually associated with mastitis. The assays of the invention comprise providing a test sample of milk from tissue suspected of being inflamed. The SAA level in the sample is measured, and preferably compared to a milk sample taken from known normal tissue. The amount of increase in SAA in the test sample as compared to the control sample is directly proportional to the level of inflammation present in the tissue from which the sample was taken.

SAA exists in several isoforms, most of which are increased in production in an inflammatory response. In accordance with the present invention, any one inflammation-responsive isoform, or a combination of isoforms, may be detected in the assay. In a preferred embodiment applicable to testing cow's milk, bovine SAA isoforms are detected. One isoform of bovine SAA has been reported to date. However, in accordance with the present invention, a new bovine SAA isoform has been

identified, which is associated with colostrum and which may comprise part or all of the SAA found in milk from inflamed tissue. Accordingly, the present invention contemplates detecting one or both of these SAA isoforms in assays of cow's milk. A portion of the amino acid sequence comprising the bovine colostrum-associated SAA is set forth herein as SEQ ID NO:1. A portion of the amino acid sequence of the bovine serum SAA isoform is set forth herein as SEQ ID NO:2.

10 In alternative embodiments, isoforms of SAA from different species are detected (partial amino acid sequences of these isoforms are set forth herein). These include, but are not limited to, human SAA1 (SEQ ID NO:3), human SAA3 (SEQ ID NO:4), rabbit SAA1 (SEQ ID NO:5), rabbit SAA3 (SEQ ID NO:6), mouse SAA1 (SEQ ID NO:7), mouse SAA3 (SEQ ID NO:8), hamster SAA1 (SEQ ID NO:9), hamster SAA3 (SEQ ID NO:10), horse SAA (SEQ ID NO:11), horse colostrum-associated SAA (SEQ ID NO: 12), pig colostrum-associated SAA (SEQ ID NO: 13), mink SAA1 (SEQ ID NO:14) and dog SAAa (SEQ ID NO:15).

Any assay that detects and quantifies SAA in a sample is contemplated for use in the present invention. Two categories of assays are preferred. One is an immunological assay for SAA protein and the other is a hybridization assay for mRNA encoding SAA protein. The latter assay is effective because SAA is transcriptionally regulated. Accordingly, the inflammation response comprises an increase in SAA mRNA production and a concomitant increase in protein production.

Immunological assays for SAA require antibodies immunologically specific for one or more SAA isoforms. Polyclonal or monoclonal antibodies directed toward SAA may be prepared according to standard methods. Monoclonal antibodies may be prepared according to

general methods of Köhler and Milstein, following standard protocols. In a preferred embodiment, anti-SAA antibodies are raised in animals that produce little or no SAA themselves, since SAA is more immunogenic to these animals. One preferred animal source of anti-SAA antibodies is the rat. In another preferred embodiment, antibodies are prepared that react with a plurality of SAA isoforms. Such antibodies may be polyclonal or monoclonal antibodies. In a particularly preferred embodiment, antibodies such as those described by McDonald et al. (J. Immunol. Meth. 144: 149-155, 1991) are used. These antibodies, raised against human SAA, also react with a variety of SAA isoforms from other species, including bovine SAA.

A variety of immunological assays known in the art are available to detect and quantitate SAA in milk samples or samples of other secreted biological fluids. These include, but are not limited to, (1) immunoprecipitation followed by protein quantification; (2) immunoblot analysis (e.g., dot blot, Western blot) (3) radioimmune assays, (4) nephelometry, turbidometric or immunochromatographic (lateral flow) assays, and (5) enzyme-coupled assays, including ELISA and a variety of qualitative rapid tests (e.g., dip-stick and similar tests). Of these, ELISA assays are preferred for use in the invention, due to their ease and economy of use, and their portability for use in the field.

ELISA assays have been developed and used for measuring the amount of SAA in a sample of blood. These same assays, or variations thereof as devised by one skilled in the art, may be used in the assays of the present invention. Such assays are described in detail in the art (e.g., McDonald et al., 1991, *supra*; Satoh et al., Am. J. Vet. Res. 56: 1286-1291, 1995; Wilkins et

al., Clin. Chem. 40: 1284-1290, 1994; Taktak & Lee, J. Immunol. Meth. 136: 11-16, 1991). An exemplary assay is set forth in Example 1.

Hybridization assays to measure the presence and amount of SAA-encoding mRNA in a sample require nucleic acid probes that specifically hybridize with the mRNA encoding one or more SAA isoforms. The availability of amino acid and nucleotide sequence information for a wide variety of SAAs enables the design and production of such probes, according to known methods. In one embodiment, the probes may be designed to hybridize with an mRNA encoding a particular SAA isoform, by choosing segments of the mRNA that are unique to that isoform. In a preferred embodiment, the probes are designed to hybridize to a portion of the SAA-encoding mRNA that is conserved across isoforms, thereby generating a probe that detects a plurality of SAA isoform mRNAs in the test sample.

As is known in the art, suitable hybridization probes comprise single stranded DNA or RNA molecules. Preferably they are between 10 and 200 nucleotides in length, and most preferably between 18 and 100 nucleotides in length. The probes may be synthesized with a detectable label; alternatively, they may be detectably labeled after synthesis. Probes also may be obtained from cloned DNA that encodes SAA.

Methods in which the aforementioned nucleic acids may be utilized as probes for hybridization assays include, but are not limited to: (1) dot blot hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR). Preferred for use in the present invention are rapid hybridization assay, such as dot blots or portable PCR-based assays. These assays are suitable for use in a clinical or field environment. Such assays are described in detail in the

art (see, e.g., "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1999).

The assays of the invention are of great utility and value in a variety of applications. Focusing on the exemplary embodiment of the inventions, the assays are particularly suitable for use in early and localized detection of mammary inflammation associated with mastitis. Expanding on this utility, the assays can also be used to monitor the therapeutic effectiveness of treatment of mastitis. The treatment may comprise an established treatment. Alternatively, the assays can be used to screen and evaluate the effectiveness of novel therapeutic agents and treatment regimens. In this embodiment, the milk of control and test animals (subjected to the new treatment or agent) are assayed for the presence and amount of SAA. A decrease in SAA in the test sample, as compared to the control sample, is indicative that the new therapeutic agent or treatment is effective in controlling the inflammation.

The assays described above also may be used to determine if a therapeutic or other agent applied to udder tissue causes any detrimental inflammatory side effects. In this embodiment, as above, milk samples from test animals receiving the treatment is compared with milk samples from control animals for the presence and amount of SAA. In this embodiment, an increase in SAA level in the test samples as compared to the control sample indicates that the treatment has a detrimental inflammatory side effect.

The assays of the invention also may be used to monitor milk quality. A current practice in many parts of the world is to assign a quality rating to a milk batch. Thus, a batch of milk being sold by a farmer is currently assessed for quality by measuring the number of somatic cells present in a sample of the milk, which is

correlated with the prevalence of clinical and sub-clinical mastitis in the dairy herd. The assays of the present invention provide a rapid, convenient alternative to the somatic cell measurement to evaluate the quality of a batch of milk.

As another useful embodiment, since SAA is elevated in colostrum and not in milk from normal breast tissue, the measurement of SAA in a milk sample can be used to differentiate colostrum from milk. Accordingly, in instances where it is undesirable to have milk that contains colostrum (some countries have laws to this effect), the methods of the invention may be used to detect colostrum-tainted milk.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

#### EXAMPLE 1

##### Immunoassay for SAA

#### 1. Preparation of Microtiter Plate:

96-well microtiter plates were prepared by incubating each well with 100  $\mu$ l of rat monoclonal antibody to bovine SAA (titer = 5.0  $\mu$ g/ml) in carbonate buffer (0.015 M  $\text{Na}_2\text{CO}_3$ , 0.035 M  $\text{NaHCO}_3$  and a pH of 9.6). This plate coating antibody is designated C-100-8. Plates were incubated for 12 hours at 4° C then the antisera solution is aspirated and the plates washed by filling and emptying all wells 4 times with PBS-Tween which consists of 0.05% v/v Tween 20 (polyoxyethylene sorbitan monolaurate) in phosphate buffered saline (PBS=0.010 M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.003 M  $\text{KH}_2\text{PO}_4$ , 0.132 M  $\text{NaCl}$ , pH of 7.2). Plates were treated with StabilCoat (SurModics Inc., Eden Prairie, MN) for 1 hour at 22°C, dried for 8 hours at 37° C, then sealed in foil bags.

## 2. Reference standard:

A solution containing known standard concentration of bovine SAA at 37.5 ng/ml was further diluted two-fold serially 6 times in PBS-Tween. Fifty microliters (50 $\mu$ l) of the standard and each of the subsequent 6 serial dilutions were used on the plate to establish a standard curve. The standard contained 37.5 ng/ml and the subsequent two-fold dilutions contained 18.8, 9.4, 4.7, 2.3, 1.20 and 0.60 ng/ml, respectively. Fifty  $\mu$ l of PBS-Tween without SAA established background readings.

## 3. Assay procedure (standard sandwich ELISA):

a. Wells were washed three times with PBS-Tween wash buffer. After the last wash, wells were patted dry on absorbent paper.

b. 50 $\mu$ l of biotinylated rat monoclonal antibody were added to bovine SAA (1  $\mu$ g/ml) to each well. This antibody is designated C-100-7 and has a different epitope specificity for SAA than the plate-coating antibody.

c. Serum, colostrum or milk samples were at room temperature and were vortexed vigorously before dilutions were made. Samples were diluted 1:500. In some instances, samples may require greater dilution to fall within the range of the assay. 50 $\mu$ l of the diluted sample or standard was added to each well. Sides of the plate were tapped to mix gently.

d. The plate was covered with a dust cover, and incubated for 1 hr at 37 $^{\circ}$ C.

e. After incubation, the plate was aspirated or decanted, then washed three times with PBS-Tween buffer. After the last wash, the plate was patted dry on absorbent paper.

f. 100 $\mu$ l of streptavidin-peroxidase (100 ng/ml) was added to each of the wells.



g. The plate was covered and incubated at room temperature in the dark for 30 minutes.

h. The wells were aspirated or decanted, then washed three times with PBS-Tween, and the plate patted dry after the last wash.

i. 100 $\mu$ l of substrate consisting of 3, 3', 5, 5' - tetramethyl benzidine at 10  $\mu$ g/ml (0.1 mg dissolved in 0.1 ml of dimethylsulfoxide and 9.9 ml of 0.1 M sodium acetate pH 6.0 added) was added.

j. The plate was covered and incubated in the dark at room temperature for 30 minutes.

k. 50 $\mu$ l of a Stop Solution of H<sub>2</sub>SO<sub>4</sub> (prepared by adding 2.8 ml of concentrated H<sub>2</sub>SO<sub>4</sub> to 97.2 ml of water) was added.

l. The absorbance of each well was read at 450 nm having blanked the plate reader against a chromagen blank composed of 100 $\mu$ l of TMB substrate and 50 $\mu$ l Stop Solution only.

m. The absorbance of the standards was plotted against standard concentration on graph paper. The background absorbance for the 0 ng/ml may be subtracted from each of the data points, including the standards, unknowns and controls prior to plotting.

n. The concentrations of the test samples and controls was determined from the standard curve by multiplying the interpolated value by the appropriate dilution factor (e.g., colostrum or milk diluted 500 should be multiplied by 500). Samples which have a signal greater than the highest standard were further diluted in diluent buffer and re-analyzed.

## EXAMPLE 2

### Evaluation of SAA in Colostrum and Subsequent Serial Samplings of Milk

The purpose of this study was to evaluate colostrum and subsequent serial milk samplings to

determine SAA content. Samples were obtained from Holstein dairy cows at the University of Nebraska - Lincoln Dairy Research Facility. Samples of colostrum were taken at calving, and subsequent milk samples were  
 5 taken twice weekly for three weeks. Samples from all four udder quadrants were pooled. Results are shown in Table 2.

Table 2: SAA Levels in Colostrum and Milk Samples

10

	<u>Cow ID</u>	<u>Sample Day</u>	<u>SAA ug/ml</u>
	83 colostrum	Calving	184.8
	83 milk	+4	0.2
	83 milk	+7	0.0
15	83 milk	+11	0.0
	83 milk	+14	0.0
	83 milk	+18	0.0
	83 milk	+21	0.0
20	932 colostrum	Calving	566.2
	932 milk	+4	229.8
	932 milk	+7	217.7
	932 milk	+11	169.0
	932 milk	+14	6.4
25	932 milk	+18	5.8
	932 milk	+21	26.0
	908 colostrum	Calving	135.6
	908 milk	+4	2.6
30	908 milk	+7	9.1
	908 milk	+11	8.2
	908 milk	+14	2.0
	908 milk	+18	2.1
	908 milk	+21	3.6
35	961 colostrum	Calving	364.6

	961 milk	+4	0.3
	961 milk	+7	0.5
	961 milk	+11	0.0
	961 milk	+14	0.0
5	961 milk	+18	0.0
	961 milk	+21	0.0

The results obtained above for cows 83, 908 and 961, each of which was free of mastitis in all quadrants, shows the low basal level of SAA present in normal milk samples after colostrum has cleared.

The results obtained for cow 932 demonstrates that SAA level can effectively detect mastitis in a quadrant specific manner. Midway through the sampling period, cow 932 was observed to be symptomatic for mastitis in one quadrant. Even though all four quadrants' milk were pooled in the first three milk samplings, the samples were elevated in SAA. In the subsequent samplings, the mastitis quadrant was not milked, so was not pooled with the milk from the unaffected quadrants. As a result, SAA levels decreased in those samples to approximately the levels observed in the completely asymptomatic cows.

25

### EXAMPLE 3

#### SAA Levels as a Quadrant-Specific Indicator of Mastitis

The purpose of this study was to determine if SAA can detect mastitis-related inflammation, and if SAA is elevated only in the affected quadrant, or in milk from all quadrants.

Samples were obtained from the Holstein dairy herd at the University of Nebraska - Lincoln Dairy Research Facility. Three cows with mastitis in the left rear (LR) quadrant of the udder were selected. Each cow

was judged visually for the level of inflammation present in each cow. Cow 874 displayed low-grade inflammation, while cow 59 displayed significant inflammation and cow 978 displayed high grade inflammation.

5 Milk samples were taken from all four quadrants of each cow, and SAA levels were determined by ELISA for the non-pooled samples. Results are shown in Table 3.

**Table 3: SAA Levels in Milk from Udder Quadrants**

10

	Cow ID	Quadrant	SAA (ug/ml)
	874	RF	12
	874	RR	10
	874	LF	11
15	874	LR (Mastitis)	73
	59	RF	1
	59	RR	1
	59	LF	7
	59	LR (Mastitis)	212
20	978	RF	6
	978	RR	18
	978	LF	8
	978	LR (Mastitis)	2368

25

The results demonstrate that SAA levels provide a quadrant-specific indicator of inflammation due to mastitis. SAA elevation is confined to the affected quadrant. Moreover, a direct correlation was observed  
 30 between the visual symptoms of inflammation and SAA levels found in the affected quadrant.

The present invention is not limited to the embodiments described above, but is capable of  
 35 modification within the scope of the appended claims.

We claim:

1. A method for detecting an inflammatory response in a breast of a lactating mammal, which comprises measuring the presence or amount of a Serum Amyloid A (SAA) protein or mRNA encoding the protein, in a sample of milk obtained from the breast, the amount of the SAA protein or mRNA present in the sample being positively correlated with the inflammatory response.

2. The method of claim 1, wherein the mammal is the inflammatory response is mastitis.

3. The method of claim 2, wherein the mastitis is caused by infection with a microorganism.

4. The method of claim 2, wherein the inflammatory response is caused by a non-infective agent.

5. The method of claim 1, wherein the SAA comprises one or more inflammation-responsive isoforms of SAA.

6. The method of claim 5, wherein the SAA comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:1-15.

7. The method of claim 1, which comprises measuring the amount of SAA protein in the milk sample.

8. The method of claim 7, wherein the amount of SAA protein is measured using an immunological assay with antibodies immunologically specific for one or more isoforms of SAA.

9. The method of claim 8, wherein the immunological assay is an ELISA assay.

10. The method of claim 1, which comprises measuring the amount of SAA mRNA in the milk sample.

11. The method of claim 10, wherein the amount of SAA mRNA is measured using a hybridization assay with nucleic acid molecules complementary to the SAA mRNA.

12. A diagnostic kit for screening milk samples to detect inflammation in breasts of lactating mammals, which comprises a container containing one or more antibodies immunologically specific for one or more SAA isoforms, and instructions for performing immunological assays of milk samples for SAA, using the antibodies.

13. The kit of claim 12, which further comprises at least one additional reagent for performing the immunological assays.

14. A diagnostic kit for screening milk samples to detect inflammation in breasts of lactating mammals, which comprises a container containing one or more nucleic acid molecules that specifically hybridizes to mRNA encoding or more SAA isoforms, and instructions for performing hybridization assays of milk samples for SAA, using the nucleic acids.

15. The kit of claim 14, which further comprises at least one additional reagent for performing the hybridization assays.

16. A method to assess the ability of a treatment or agent to increase or decrease inflammation in breast tissue, which comprises:

- (a) obtaining a milk sample from a test subject to which the treatment or agent has been administered;
- (b) obtaining a milk sample from a control subject which has not been administered the treatment or agent;

(c) measuring the amount of SAA in the test sample and the control sample; and

(d) comparing the measured amounts of SAA, an increase in SAA in the test sample as compared to the control sample indicating the ability of the treatment or agent to increase inflammation in the breast tissue, and a decrease in SAA in the test sample as compared to the control sample indicating the ability of the treatment or agent to decrease inflammation in the breast tissue.

17. The method of claim 16, adapted for screening anti-microbial agents for treatment of mastitis.

18. A method of evaluating quality of a milk sample, which comprises:

(a) establishing a standard that correlates a pre-determined milk quality with a pre-determined concentration of SAA contained therein;

(b) measuring SAA concentration in the milk sample being tested for quality;

(c) comparing the amount of SAA in the milk sample with the established standard; and

(d) assigning a quality rating to the milk sample based on the comparison with the standard.

19. A method of determining the presence or amount of colostrum in a milk sample, which comprises:

(a) measuring SAA concentration in a milk test sample suspected of containing colostrum;

(b) comparing the amount of SAA in the test sample with an amount of SAA in a control milk sample known to contain no colostrum, an elevation in SAA in the test sample as compared with the control sample being indicative of the presence and amount of colostrum in the test sample.

20. The method of claim 19, which further comprises measuring a colostrum-specific isoform of SAA.

b) comparing the amount of SAA in the test sample with an amount of SAA in a control milk sample known to contain no colostrum, an elevation in SAA in the test sample as compared with the control sample being  
5 indicative of the presence and amount of colostrum in the test sample.

21. The method of claim 20, which further comprises measuring a colostrum-specific isoform of SAA.



## SEQUENCE LISTING

<110> McDonald, Thomas L.  
Weber, Annika

<120> Diagnostic Assays of Secreted Biological  
Fluids for Detection of Infection and Inflammatory  
Conditions

<130> UNMC 98018A

<160> 15

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 20

<212> PRT

<213> Cow

<400> 1

Met	Trp	Xaa	Thr	Phe	Leu	Lys	Glu	Ala	Gly	Gln	Gly	Ala	Lys	Asp	Met
1				5					10					15	
Trp	Arg	Ala	Tyr												
			20												

<210> 2

<211> 27

<212> PRT

<213> Cow

<400> 2

Xaa	Xaa	Trp	Met	Ser	Phe	Phe	Gly	Glu	Ala	Tyr	Glu	Gly	Ala	Lys	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Arg	Glu	Ala					
			20					25							

<210> 3

<211> 27

<212> PRT

<213> Homo sapiens

<400> 3

Arg	Ser	Phe	Phe	Ser	Phe	Leu	Gly	Glu	Ala	Phe	Asp	Gly	Ala	Arg	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Arg	Glu	Ala					
			20					25							

<210> 4

<211> 27

<212> PRT

<213> Homo sapiens

2

&lt;400&gt; 4

Gln	Gly	Trp	Leu	Thr	Phe	Leu	Lys	Ala	Ala	Gly	Gln	Gly	Ala	Lys	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Lys	Glu	Ala					
			20					25							

&lt;210&gt; 5

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Rabbit

&lt;400&gt; 5

Gln	Arg	Trp	Phe	Ser	Phe	Ile	Gly	Glu	Ala	Thr	Gln	Gly	Ala	Trp	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Arg	Glu	Ala					
			20					25							

&lt;210&gt; 6

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Rabbit

&lt;400&gt; 6

Arg	Glu	Trp	Leu	Thr	Phe	Leu	Lys	Glu	Ala	Gly	Gln	Gly	Ala	Lys	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Lys	Glu	Ala					
			20					25							

&lt;210&gt; 7

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 7

Gly	Gly	Phe	Phe	Ser	Phe	Val	His	Glu	Ala	Phe	Gln	Gly	Ala	Gly	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Thr	Asp	Met	Lys	Glu	Ala					
			20					25							

&lt;210&gt; 8

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 8

Gln	Arg	Trp	Val	Gln	Phe	Met	Lys	Glu	Ala	Gly	Gln	Gly	Ser	Arg	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Lys	Lys	Ala					
			20					25							

&lt;210&gt; 9

&lt;211&gt; 27

&lt;212&gt; PRT

<213> Hamster

<400> 9

Gln	Arg	Trp	Phe	Gln	Phe	Met	Lys	Glu	Ala	Gly	Gln	Gly	Thr	Arg	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Thr	Asp	Met	Arg	Glu	Ala					
			20					25							

<210> 10

<211> 27

<212> PRT

<213> Hamster

<400> 10

Gln	Arg	Trp	Phe	Gln	Phe	Met	Lys	Glu	Ala	Gly	Gln	Gly	Ser	Thr	Asp
1				5					10					15	
Met	Trp	Arg	Ala	Tyr	Ser	Asp	Met	Arg	Glu	Ala					
			20					25							

<210> 11

<211> 27

<212> PRT

<213> Horse

<400> 11

Xaa	Xaa	Leu	Leu	Ser	Phe	Leu	Gly	Glu	Ala	Ala	Arg	Gly	Thr	Trp	Met
1				5					10					15	
Asp	Leu	Arg	Ala	Thr	Asn	Asp	Met	Arg	Glu	Ala					
			20					25							

<210> 12

<211> 13

<212> PRT

<213> Horse

<400> 12

Arg	Glu	Leu	Lys	Thr	Phe	Leu	Lys	Glu	Ala	Gly	Gln	Gly
1				5					10			

<210> 13

<211> 10

<212> PRT

<213> Pig

<400> 13

Trp	Leu	Leu	Thr	Phe	Leu	Lys	Glu	Ala	Gly
1				5				10	

<210> 14

<211> 27

<212> PRT

<213> Mink

<400> 14  
Gln Xaa Trp Tyr Ser Phe Ile Gly Glu Ala Ala Gln Gly Ala Trp Asp  
1 5 10 15  
Met Tyr Arg Ala Tyr Ser Asp Met Ile Glu Ala  
20 25

<210> 15  
<211> 27  
<212> PRT  
<213> Dog

<400> 15  
Gln Xaa Trp Tyr Ser Phe Val Gly Glu Ala Ala Gln Gly Ala Trp Asp  
1 5 10 15  
Met Leu Arg Ala Tyr Ser Asp Met Arg Glu Ala  
20 25

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/19418

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : 435/4, 6, 7.1, 7.2, 7.72, 7.9, 7.92, 7.93, 7.94, 7.95, 34.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 7.1, 7.2, 7.72, 7.9, 7.92, 7.93, 7.94, 7.95, 34.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,425,330 A (NORCROSS et al.) 10 January 1984, col. 5, line 60-col. 8, line 36 .	1-5, 7-21
Y	US 5,536,640 A (SIPE et al.) 16 July 1996, col. 6, line 65-col. 11, line 46.	1-5, 7-21
A	US 4,377,569 A (PLYMATE) 22 March 1983, col. 1, line 52-col. 4, line 5.	1-5, 7-21
A	US 5,700,465 A (TAO et al.) 23 December 1997, col. 1, line 45-col. 5, line 42.	1-5, 7-21
A	US 5,585,098 A (COLEMAN) 17 December 1996, col. 4, line 65-col. 7, line 44.	1-5, 7-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

16 DECEMBER 1999

Date of mailing of the international search report

27 JAN 2000

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

PENSEE T. DO



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/19418**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 6  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Claim 6 recites SEQ ID NO's and the CRF for the application is defective.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/JS99/19418

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (7):

C12Q 1/00, 1/04, 1/68; G01N 33/53, 33/567, 33/542, 33/537, 33/543.

**THIS PAGE BLANK (USPTO)**